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This project aims to increase the transduction efficiency and target cell-specificity of retroviral vectors, thereby improving gene transfer to prostate cancer cells. We have developed a novel replication-competent retroviral (RCR) vector system for gene transfer to solid tumors which is highly efficient, as each tumor cell which is successfully transduced becomes itself a virus-producing cell and initiates further infection events even after the initial injection. Through this project, we have tested a number of strategies for targeting these replicating vectors specifically and exclusively to tumor cells in order to limit and control the replicative process and minimize the risk to normal cells. We have engineered the prostate-specific probasin promoter into the retroviral long terminal repeat (LTR), and used this construct to establish producer cell lines derived from prostate cancer cells that express androgen receptors. We have optimized the design of the probasin/LTR hybrid promoter construct, and we have demonstrated the prostate-specificity of probasin-targeted RCR vector replication and suicide gene transfer in culture and *in vivo*.

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N. Takahara

July 15, 2003

PI - Signature **Date**

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Attachment (publication):

Logg, C. R., Logg, A. , Matusik, R., Bochner, B. H., Kasahara, N.
Tissue-specific targeting of replication-competent retrovirus vectors by transcriptional regulation.
Journal of Virology, 76: 12783-12791, 2002.

Final Report **July 15, 2003**

I. INTRODUCTION

Currently, one of the foremost obstacles to the practical implementation of gene therapy is the low transduction efficiency of gene delivery vectors *in vivo*. This project aims to improve gene transfer to prostate cancer through the development of replication-competent retrovirus (RCR) vectors that are transcriptionally targeted to prostate cancer cells.

We have devised a novel construct design to produce replicating retrovirus vectors that have proven to be stable over multiple serial passages in cell culture, and that are capable of highly efficient gene delivery to cancer cells *in vivo* in a solid tumor xenograft model in nude mice. As active cell division is required for retroviruses to gain access to the nucleus and integrate into the host chromosomes, these vectors already show relative selectivity for actively dividing cancer cells. In this project, our aim is to further enhance the specificity and safety of this approach by incorporation of suicide genes into the vector and by replacing the long terminal repeat (LTR) promoter of the retrovirus with transcriptional control elements from the highly prostate-specific probasin promoter, thus limiting replication of the viral genome to prostate cancer cells, and thereby avoiding inadvertent spread into surrounding normal tissues.

II. BODY

During the period from January 15, 2000 to July 14, 2003, we have substantially accomplished the Tasks listed in the approved Statement of Work, shown below.

Statement of Work:

Task 1. Construction of RCR vectors targeted to human prostate cancer cells (Months 1–8)
Task 2. Creation of RCR vector-producing cell lines (Months 8–14)
Task 3. Testing the tissue specificity of the virus in culture (Months 14–21)
Task 4. Transduction of human prostate cancer cells *in vivo* (Months 21–36)

Research accomplishments:

***Task 1.* Construction of RCR vectors targeted to human prostate cancer cells (Months 1–8)**

We have applied the strategy of targeting specific cell types via promoter modification to replication-competent retroviral (RCR) vectors, for gene therapy of prostate cancer. Directing RCR vectors specifically to prostate cancer cells provides a way to limit the spread of virus while enabling more efficient transduction of tumors.

Results:

Construction of hybrid probasin-MLV LTRs

Highly prostate-specific promoter elements have previously been utilized to achieve prostate-specific transgenic targeting of various oncogenic proteins (Greenberg et al., 1995; Greenberg et al., 1994). One of the most well-characterized proteins uniquely produced by the prostate and stringently regulated by prostate-specific promoter sequences, is the rat probasin protein. A fragment of the probasin promoter sequence, containing bases -426 to +28 of the 5' untranslated region, has been extensively studied in CAT reporter gene assays, and prostate-specific expression in transgenic mouse models using this promoter has been reported (Greenberg et al., 1994). Gene expression levels in these models parallel the sexual maturation of the animals with 70-fold increased gene expression found at the time of puberty (2-6 weeks). Castration of the animals will drop gene expression to near zero which can be increased to pre-castrate levels following the parenteral administration of testosterone. Two independent groups have used the –426 to +28 fragment of the probasin promoter for targeted overexpression of the SV40 T antigen gene in the prostate, resulting in the establishment of transgenic mouse models of prostate cancer (TRAMP mice) (Greenberg et al., 1994) which recapitulate many of the phenotypic features of human disease, including androgen dependent growth and distant site metastasis.

We have therefore constructed and tested a number of RCR vector constructs driven by probasin/MLV hybrid LTR promoters using various design strategies. These new constructs are based on both the wild type probasin (PB) promoter fragment described above, as well as a more potent synthetic probasin promoter construct (designated ARR_2PB) obtained from Dr. Robert Matusik (Vanderbilt University). The proximal probasin promoter contains *cis* elements that direct prostate-specific transcription, including a 148-bp sequence from -244 to -96 called the androgen responsive region (ARR, Fig. 1A). Within the ARR are two androgen receptor binding sites, which work cooperatively in the induction of promoter activity by androgens. The synthetic variant of the proximal PB promoter constructed by Dr. Matusik, ARR_2PB, contains two copies of the ARR and exhibits more robust transcriptional activity than the wt promoter *in vitro* and *in vivo* (Zhang et al, 2000).

The wild type PB promoter, the synthetic ARR_2PB promoter, and the MLV U3 region promoter each contain CAAT and TATA boxes. Therefore, a series of six hybrid probasin/MLV LTR constructs were generated by replacement of the MLV U3 sequence from near the 5' end of the LTR to either the CAAT box, TATA box, or transcription start site, with the corresponding upstream sequences from either the wild type PB promoter (Fig. 1B) or the ARR_2PB promoter (Fig. 1C). Each of these LTRs was designed such that transcription would be initiated at the 5' border of the R region, as occurs in wt MLV.

Our hypothesis was that some of the original MLV LTR sequences may need to be retained for the virus to replicate successfully; for example, as the LTRs must provide both promoter and polyadenylation functions, the TATA box sequence in the MLV LTR promoter is also thought to overlap a polyadenylation signal. Thus, specific sequences inherent in the original MLV LTR may be important for this dual role and hence for optimal replication of the virus.

The 5' end of the U3 region is recognized by viral integrase protein, and so overlap extension PCR was used to precisely place the probasin promoter just downstream of the beginning of the U3 region in the viral LTR, replacing the rest of the U3 sequence up to the R region, where transcription is initiated in the original LTR; these hybrid LTRs, designated Pr and Ar, contain the wt PB promoter and ARR_2PB, respectively. The next set of hybrid LTRs, designated Pt and At, contain the wt PB promoter and ARR_2PB, respectively, from their 5' ends to the 5' end of their TATA boxes, fused at the MLV TATA box. Finally, hybrid LTRs Pc and Ac contain the wt PB promoter and ARR_2PB, respectively, from their 5' ends to the 5' end of their CAAT boxes, fused to the MLV CAAT box (Fig. 1D).

Using these chimeric probasin/LTR constructs, a series of six reporter plasmids in which each the hybrid LTRs controls expression of the luciferase cDNA was generated (Fig. 1E). These reporter plasmids were first employed in transient transfection assays using a variety of prostate cancer cell lines and non-prostatic cell lines to confirm the transcriptional specificity of each hybrid promoter sequence.

Subsequently, these six hybrid LTR constructs were engineered into the retroviral 3' LTR in both ecotropic and amphotropic RCR vectors. Since it is initially placed downstream, this modified U3 region will not be operative upon transfection of the provirus construct into 293 cells and production of the vector transcript will proceed normally. However, after a single round of replication the probasin sequence will be re-duplicated in the U3 region of the 5' LTR (Fig. 2), and thereafter should specify prostate cell-specific replication of the virus.

It should be noted in this context that, although insertions of non-essential transgenes in the U3 region are prone to deletion, the probasin promoter in this case will completely replace the wild type promoter elements in the viral LTR, therefore deletions of the probasin promoter would simply result in a virus that is unable to replicate, and we hypothesized that there would be selection pressure against such deletions.

We have thus successfully constructed probasin-targeted RCR vectors containing the GFP marker gene. The probasin-targeted vector carrying the PNP suicide gene was also constructed similarly (see below). To our knowledge, this represents the first example of a replicating MLV vector controlled by transcriptional regulation (Logg et al, *J Virol* 2002).

Figure 1: Structure of hybrid probasin-LTRs.

Constructs used in this study. (A) Sequences used in generating hybrid LTRs. The proximal rat PB promoter (left) contains CAAT and TATA box homologies and an ARR (shown as a hatched box) important for androgen induction of transcription. ARR_2PB (center) is a synthetic variant of the PB promoter and contains two copies of the ARR. The MLV LTR (right) comprises the U3, R, and U5 regions. The transcriptional control sequences of MLV are located primarily in the U3 region, which also contains CAAT and TATA box sequences. (B) Hybrid LTRs containing the wt PB promoter. LTRs Pr, Pt, and Pc contain PB promoter sequences from position -383 to the transcription start site (TSS), TATA box, and CAAT box, respectively. (C) Hybrid LTRs containing ARR_2PB. LTRs Ar, At, and Ac contain ARR_2PB sequences from the 5' end of the upstream ARR to the TSS, TATA box, and CAAT box, respectively. In each of the six hybrid LTRs, MLV U3 sequences from the *Nhe*I site to the TSS, TATA box, or CAAT box were replaced with the corresponding PB or ARR_2PB sequences. (D) Sequence details of the hybrid LTRs. Shown are the nucleotide sequences at the 3' borders between the PB and MLV sequences. TATA and CAAT boxes are underlined. TSS, transcription start site. (E) Luciferase reporter constructs containing hybrid LTRs. (F) Structure of replication-competent MLV vectors containing hybrid LTRs. Each vector contains an IRES-GFP cassette positioned immediately downstream of the *env* gene and a 5' LTR in which the U3 region was replaced by the CMV immediate-early promoter. The 3' LTR is used to form the 5' LTR during MLV replication. We therefore replaced the 3' LTR of the RCR vector with the hybrid LTRs.

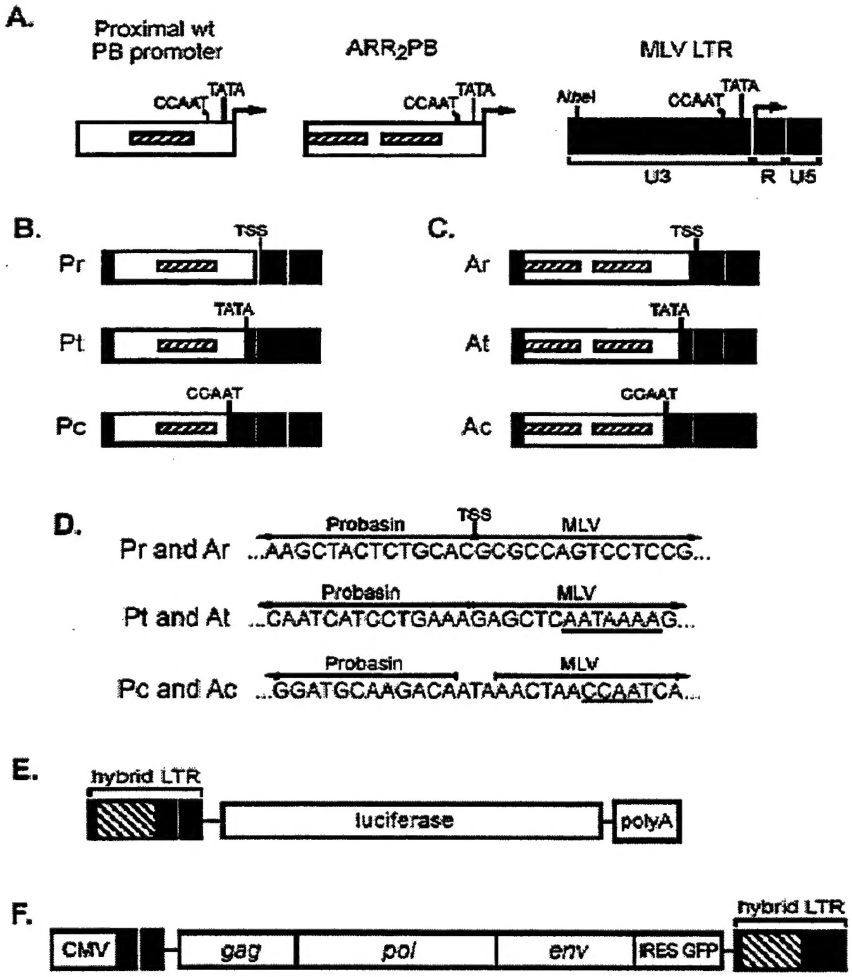
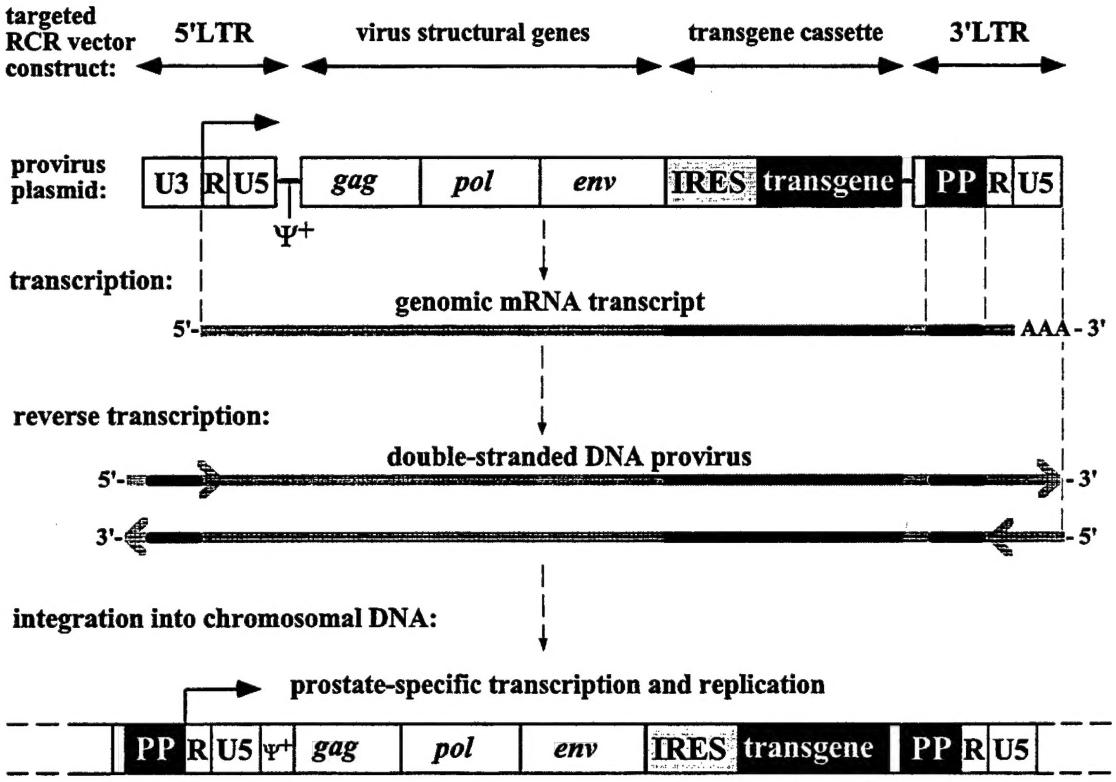


Figure 2: Design of RCR vectors targeted to prostate cancer cells.
(PP: probasin promoter, LTR: long terminal repeat, Ψ: packaging signal)



Probasin-targeted RCR vectors expressing suicide genes.

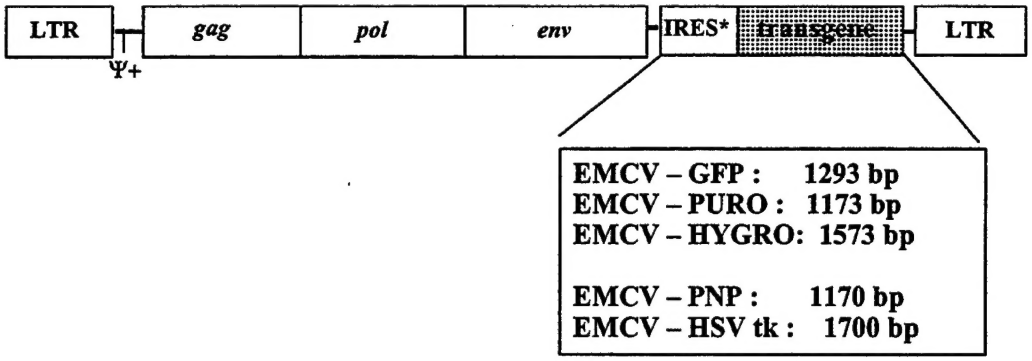
We have now also constructed and tested similar RCR vectors that contain a suicide gene, using the same construct design that links expression of the suicide gene to retroviral envelope (env) gene expression via an internal ribosome entry site (IRES).

As suicide genes, we initially proposed to test two different pro-drug-activating enzymes: 1) E. coli purine nucleoside phosphorylase (PNP), and 2) Herpes simplex thymidine kinase (HSV-tk). The E. coli PNP gene was positioned just downstream of the IRES sequence (Fig. 3) in our RCR vector constructs. The PNP gene is the same size as small marker genes such as GFP or the puromycin resistance gene, and indeed the stability of vectors containing transgenes of this size has been found to be comparable (Logg et al, *J Virol* 2001).

However, construction of vectors containing the larger HSV-tk gene was not pursued due to the results of our recent studies examining the stability of RCR vectors containing marker genes of similar size such as the hygromycin resistance gene (Fig. 3). In our original proposal we had already discussed the possibility that vectors containing larger transgenes might prove to be less stable, however we had hoped to utilize HSV-tk because it has been well characterized and frequently utilized in clinical gene therapy trials. Unfortunately, even the 1.6-kb IRES-hygromycin resistance gene construct showed extreme instability in the absence of antibiotic selection pressure (Logg et al, *J Virol* 2001), therefore we anticipate that the larger HSV-tk gene will show similar instability upon serial passage.

Figure 3: Structure of replication-competent retroviral (RCR) vectors containing an internal ribosome entry site (IRES)–transgene cassette.

LTR: long terminal repeat, Ψ+: packaging signal, EMCV: IRES from encephalomyocarditis virus, GFP: green fluorescent protein marker gene, PURO: puromycin resistance selectable marker gene, HYGRO: hygromycin resistance selectable marker gene, PNP: purine nucleoside phosphorylase suicide gene, HSVtk: Herpes simplex virus thymidine kinase suicide gene.



To confirm suicide gene functionality, the untargeted PNP-containing RCR vectors were serially passaged in NIH3T3 cells, and the titers as determined by XC assay ranged from 10^4-10^5 XC plaque-forming units per ml (pfu/ml). The functionality of the PNP transgene was also tested by determining whether sensitivity had been conferred to the pro-drug 6-methylpurine-deoxyriboside (Sigma). All cell cultures infected by the PNP-expressing RCR vectors were killed after incubation with medium containing the 6-methylpurine pro-drug (data not shown), indicating that the suicide gene was functional.

Task 2. **Creation of RCR vector-producing cell lines (Months 8–14)**

Upon confirming the efficiency of androgen-inducible transgene expression using the probasin-targeted hybrid LTR promoter constructed in Task 1, we have established RCR vector-producing cell lines.

Results:

Prostate cell-specificity and androgen-inducibility of probasin-LTR hybrid promoters

In order to confirm that the wild type (wt) and synthetic (ARR_2PB) probasin promoters would still be capable of prostate-specific, androgen-inducible expression after being incorporated into the retroviral long terminal repeat (LTR), these hybrid promoters were first used to drive expression of a luciferase reporter gene (Fig. 1E).

The murine TRAMP-C and human LNCaP and MDA PCa 2b prostate carcinoma cell lines, as well as HeLa human cervical carcinoma, NMU rat mammary carcinoma, and NIH3T3 murine fibroblast cells were transiently transfected with the reporter plasmids containing the hybrid LTRs to assess transcriptional activity and cell type-specificity. Transfections were carried out both in the presence and absence of androgen to evaluate the androgen inducibility of the hybrid LTRs. As a standard, a reporter plasmid containing the SV40 early promoter was used in parallel transfections.

In the TRAMP-C and LNCaP prostate cancer cell lines, luciferase activity driven by the three wt PB promoter-targeted LTRs increased 3-4 fold upon androgen induction, while the SV40 control promoter activity remained unchanged. More dramatically, luciferase transcription from each of the ARR_2PB-targeted LTRs increased roughly 1000-fold upon androgen induction (Logg et al, *J Virol* 2002).

In MDA PCa 2b cells, all three wt PB promoter hybrid LTRs exhibited somewhat lower strength than the SV40 promoter and were not induced by androgen, while the LTRs containing ARR_2PB were significantly more potent in these cells than those containing the wt PB promoter, driving luciferase expression 3 to 4-fold higher than the SV40 promoter. Androgen also had no significant effect on transcription from the ARR_2PB-containing LTRs in MDA PCa 2b cells.

The absence of induction in MDA PCa 2b cells was most likely due to the presence in these cells of a doubly mutated androgen receptor that exhibits greatly reduced affinity to androgen and increased affinity to other steroids compared to both the wild type androgen receptor and to the singly mutated receptor present in LNCaP cells.

In the non-prostate cell lines, activity of each of the hybrid LTRs was much lower than that of the SV40 promoter, and androgen had no discernable effect on luciferase expression (Logg et al, *J Virol* 2002). In particular, the NMU rat mammary carcinoma cells are non-prostatic, yet they express androgen receptor; thus this cell line represents a stringent test of the specificity of the probasin promoter. Luciferase assay results show that expression from the probasin promoter does not, in fact, occur at significant levels even in this situation.

These results confirm that the probasin-LTR hybrid promoter is active with androgen stimulation only in prostate cell lines expressing a functionally intact androgen receptor, whereas in non-prostatic cell lines the hybrid promoter shows little activity even in the presence of androgen stimulation.

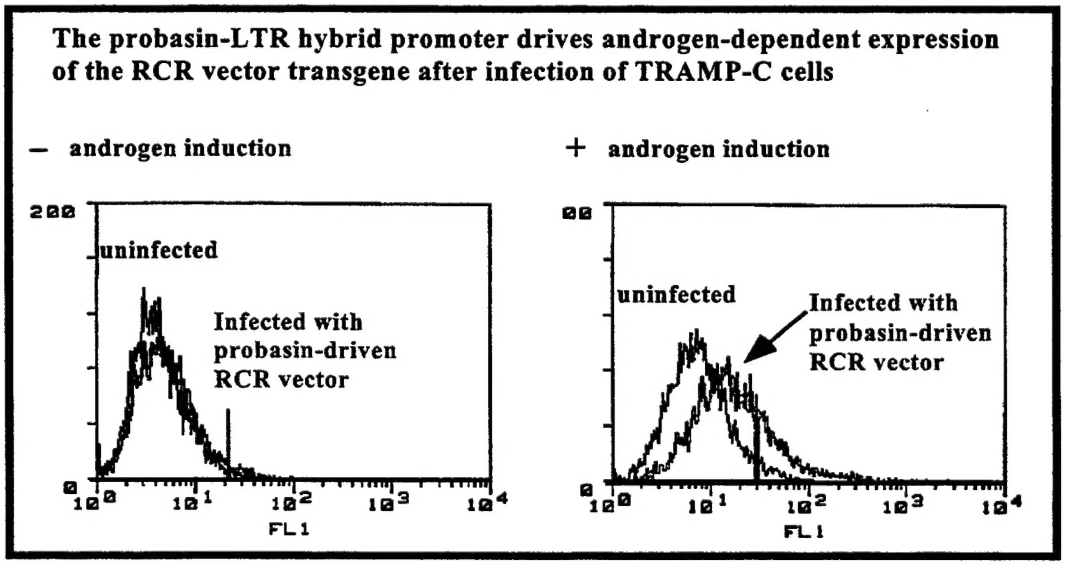
These results have been reported at national meetings and in a recent peer-reviewed publication with acknowledgment of DOD funding (Logg et al, *J Virol* 2002).

Producer lines have been established and androgen-inducible transcriptional regulation of RCR vectors driven by the probasin-LTR hybrid promoter has been achieved.

After confirming the prostate cell-specificity of the probasin-LTR hybrid promoters, initial virus preparations were generated from each of these RCR vector constructs carrying the GFP transgene, by harvesting the supernatant cell culture medium 48-72 hours after calcium phosphate-mediated transient transfection of 293T cells. These transiently produced virus preparations were filtered to exclude cell debris, and then used to infect TRAMP-C murine prostate cancer cells as well as LNCaP human prostate cancer cells, thereby establishing these as RCR producer cell lines.

As discussed above, probasin-driven expression occurs only after one round of reverse transcription and re-duplication of the 3' LTR at the 5' end (please see Fig. 2 above for details). This should have occurred upon infection of the prostate cells. Therefore, GFP expression in the prostatic producer cells was examined by fluorescence-activated cell sorter (FACS) analysis in the presence and absence of androgen stimulation. Representative results are shown in Fig. 4, demonstrating that a shift in fluorescence, indicating expression of the GFP marker gene, occurred in the prostate cancer-derived producer cells only upon androgen stimulation.

Figure 4: FACS analysis results upon androgen induction of probasin-RCR producer cells
FACS analyses were performed in the absence (left panel) or presence (right panel) of androgen, using TRAMP-C prostate cancer cells producing wt probasin/LTR-driven RCR vectors expressing GFP.



Task 3. **Testing the tissue specificity of the virus in culture (Months 14–21)**

As shown above, the androgen-inducibility of each of the probasin/LTR hybrid promoter-driven RCR vectors has been confirmed in prostate cell lines used for virus production. We then harvested vectors from these producer cells for infection and serial passage experiments to determine the specificity of probasin-driven RCR vector replication using a variety of prostate cancer cell lines versus non-prostatic control cells.

Results:

Optimal prostate-specific replication can be achieved by retaining the original TATA box sequence from MLV in the ARR_2PB probasin/MLV hybrid LTR-driven RCR vectors.

Each of the hybrid probasin/LTR-driven RCR vector constructs were then tested in various prostatic and non-prostatic cell lines, using FACS analysis to monitor the spread of the GFP transgene. The results demonstrate that efficient viral replication is achieved with the new promoter design strategies in prostate cancer cell lines, whereas little or no replication is observed in non-prostatic cell lines, including NIH3T3, HeLa, and NMU breast cancer cells. Representative results in LNCaP prostate cancer cells versus non-prostatic NMU breast cancer cells are shown in Figures 5 and 6, respectively.

Of the various new design strategies, the TATA box fusion design with the ARR_2PB probasin promoter (ACE-GFP-At) appears to show the most robust kinetics (Fig. 5), and this design also appears to show more stringent prostate-specificity, with no significant replication observed in non-prostatic cell lines (Fig. 6). In contrast, the CAAT box fusion design (ACE-GFP-Ac) appears to show slower replication kinetics in prostate cells (Fig. 5), and a low level of leaky expression in non-prostatic cells (Fig. 6).

Figure 5: ARR_2PB-targeted RCR vectors exhibit robust replication in LNCaP cells.
(A) uninfected cell control, (B) probasin-targeted RCR vector ACE-GFP-At, (C) probasin-targeted RCR vector ACE-GFP-Ac, (D) untargeted positive control RCR vector ACE-emd. Each column shows spread of GFP expression in the infected cells as demonstrated by flow cytometric analysis at the indicated time points after inoculation with vector. The vertical axis represents cell number and the horizontal axis represents fluorescence intensity.

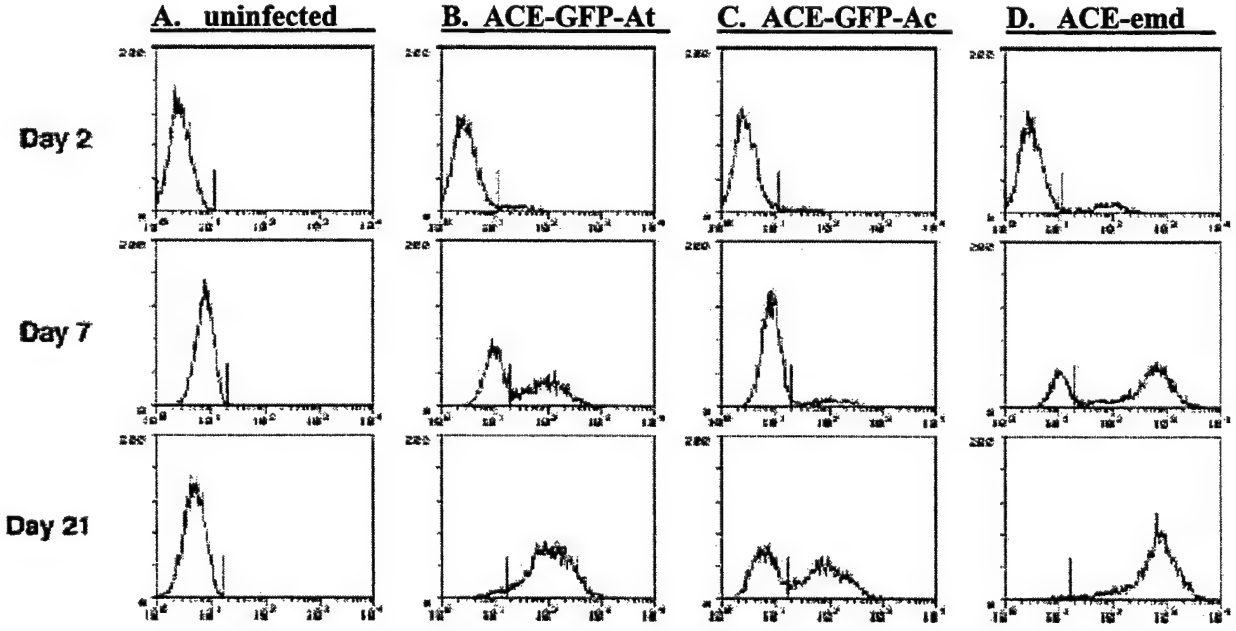
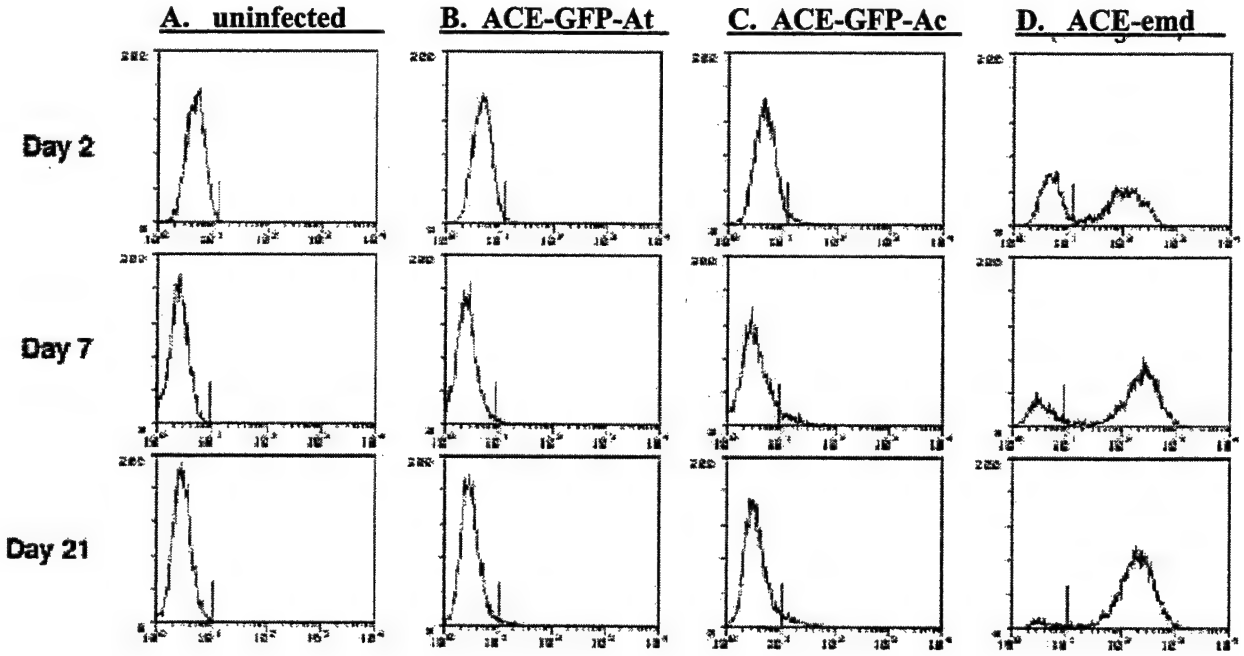


Figure 6: ARR_2PB-targeted RCR vectors show little or no replication in NMU cells.
(A) uninfected cell control, (B) probasin-targeted RCR vector ACE-GFP-At, (C) probasin-targeted RCR vector ACE-GFP-Ac, (D) untargeted positive control RCR vector ACE-emd. Each column shows spread of GFP expression in the infected cells as demonstrated by flow cytometric analysis at the indicated time points after inoculation with vector. The vertical axis represents cell number and the horizontal axis represents fluorescence intensity.



Overall it appears that probasin-driven RCR replication is well correlated with the functional androgen receptor status of the prostate cell line used, and the luciferase assay results are highly predictive of the replicative specificity of the virus for each hybrid promoter/LTR construct.

A comprehensive compilation of the results for each of the wild type and ARR_2PB probasin/LTR-driven RCR vectors in the various prostate and non-prostate cell lines has recently been published in a peer-reviewed journal with acknowledgment of DOD funding (Logg et al, *J Virol* 2002; please see Appendix).

Probasin-targeted vectors encoding the PNP suicide gene can efficiently and specifically kill prostate carcinoma cells in the presence of the pro-drug MPDR.

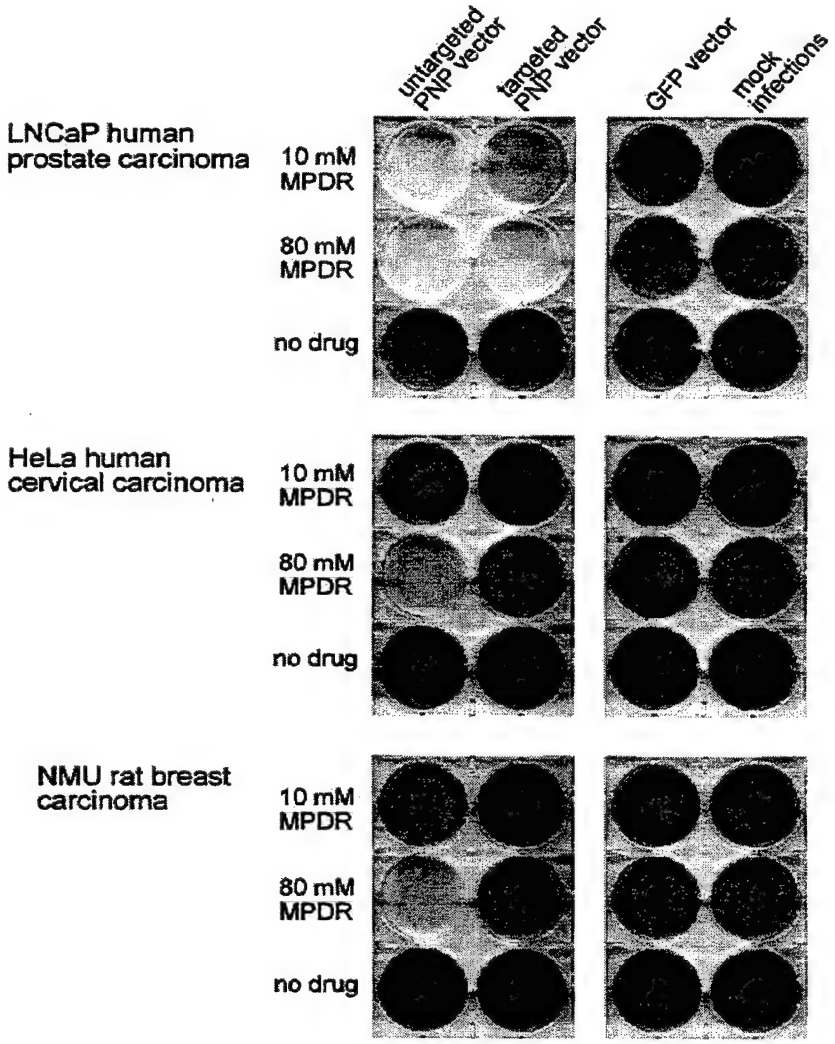
As described above, we replaced the GFP marker gene of the untargeted RCR vector ACE-GFP and the optimal ARR_2PB-targeted RCR vector ACE-GFP-At with a suicide gene encoding purine nucleoside phosphorylase (PNP) (Fig. 3), which converts the nontoxic prodrug 6-methylpurine deoxyriboside (MPDR) into the highly cytotoxic metabolite 6-methylpurine. The resulting vectors were designated ACE-PNP and pACE-PNP-At, respectively. We hypothesized that the expression of this suicide gene from the prostate-targeted replicating vector might allow the specific and efficient transduction and killing of prostate tumors, without concomitant damage to normal, nonprostatic cells in cases of prostate cancer.

LNCaP prostate and HeLa cervical carcinoma cell cultures were infected with ACE-GFP, ACE-PNP, and ACE-PNP-At and cultivated for 10 days to allow the vectors to spread. On day 10 post-infection, the pro-drug MPDR at 10 mM or 80 mM concentration was added to the culture medium. Two days later, Giemsa staining was performed to visualize remaining viable cells. As shown in Figure 7, LNCaP cells infected by either the targeted (ACE-PNP-At) or untargeted (ACE-PNP) vector encoding PNP were killed by the MPDR, while ACE-GFP-infected and mock-infected cells were not affected. In contrast, among the HeLa cultures, only those infected with ACE-PNP were killed by MPDR.

Thus the prostate-specific promoter in ACE-PNP-At was not active in HeLa cells, and unwanted cytotoxicity could be avoided in this non-prostatic cell type. These results clearly demonstrate that the replicative specificity observed with the prostate-targeted GFP-encoding replicating vector can be extended to efficient prostate-specific cell-killing using suicide genes.

Figure 7: ARR_2PB-targeted suicide gene RCR vector achieves prostate cell-specific killing.

Upper row: LNCaP human prostate carcinoma cells, middle row: HeLa human cervical carcinoma cells, lower row: NMU rat breast carcinoma cells. Cultures were stained with Giemsa to detect remaining viable cells 2 days after addition of the pro-drug 6-methylpurine deoxyriboside (MPDR) at low dose (10 mM) and high dose (80 mM) concentrations; therefore, clear wells indicate significant cell death. Infection with the positive control suicide gene RCR vector ACE-PNP (untargeted PNP vector) results in extensive cell death in all cell lines, particularly at the high dose MPDR concentration. In contrast, the ARR_2PB-targeted suicide gene RCR vector ACE-PNP-At (targeted PNP vector) efficiently kills LNCaP prostate cancer cells, but shows no cytotoxicity on either non-prostatic cell line compared to the negative control RCR vector expressing only a marker gene (GFP vector) or the uninfected cell controls (mock infections).



Task 4. **Transduction of human prostate cancer cells *in vivo* (Months 21–36)**

Animal studies have been performed using the probasin-targeted RCR vectors expressing the GFP marker gene to determine the efficiency and specificity of replicative spread in prostatic and non-prostatic tumor xenograft models *in vivo*.

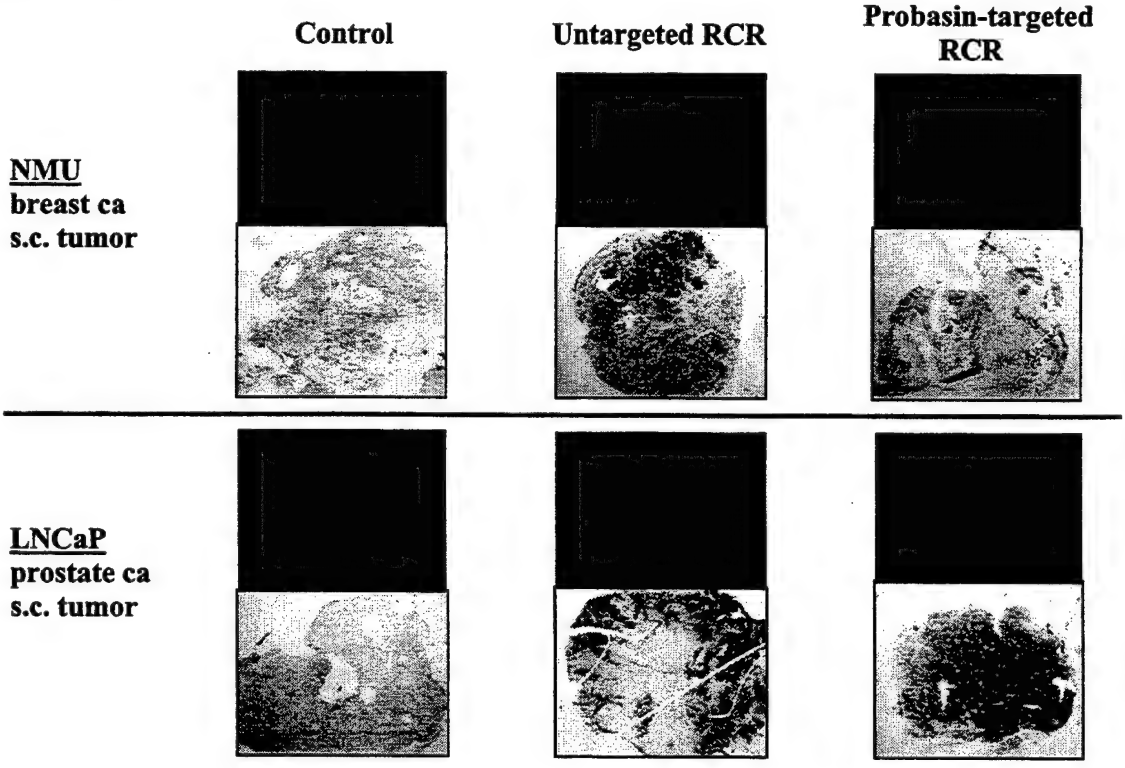
Results:

Probasin-targeted RCR vectors exhibit tumor-specific replication and highly efficient gene transfer throughout prostate cancer xenografts *in vivo*.

Tumors were established by subcutaneous injection of 2 x 10^6 LNCaP (androgen receptor-positive prostate cancer), PC-3 (androgen receptor-negative prostate cancer), NMU (breast cancer) and 5637 (bladder cancer) cells into the anterior flanks of 8-week-old nude mice. Three to four weeks later, when the tumors had grown to 1-1.5 cm in diameter, intratumoral injections of 6 x10^3 transducing units (TU) of ARR_2PB-targeted RCR vector carrying the GFP marker gene (ACE-GFP-At), non-targeted RCR vector with GFP (ACE-GFP), or saline vehicle control were performed. The subcutaneous tumors were visualized by *in vivo* fluorescence imaging, and subsequently surgically removed at the various time points and examined by immunohistochemical staining with GFP-specific monoclonal antibodies.

LNCaP tumors injected with the probasin-targeted RCR vectors were highly positive for GFP expression by both fluorescence imaging and immunohistochemistry at levels comparable to or exceeding that of the untargeted positive control RCR vector. In contrast, no GFP expression could be observed in any of the non-prostatic 5637 or NMU tumors with the targeted RCR vector, although robust replication was observed with the untargeted vector. Furthermore, as expected, androgen receptor-negative PC-3 tumors were also negative for GFP staining after infection with the probasin-targeted RCR vector, again confirming that targeted replication is highly correlated with functional androgen receptor status.

Figure 8: Probasin-targeted RCR vector exhibits prostate cell-specific replication *in vivo*.
Fluorescence imaging and immunohistochemistry of NMU tumors (upper panels) and LNCaP tumors (lower panels) injected with saline (control), ACE-GFP (untargeted RCR), or ACE-GFP-At (probasin-targeted RCR).



PCR analysis was performed on various normal organs, including brain, heart, lungs, liver, spleen, bone marrow, intestine, skin, and normal prostate, to determine whether any extratumoral spread of the probasin-targeted RCR vector might have occurred. No positive signal could be detected from any sample except for the infected prostate tumors, indicating that the RCR vector had not disseminated to normal organs (data not shown). Thus, in combination with its intrinsic inability to infect quiescent non-dividing cells, the probasin promoter-mediated targeting results in a high degree of specificity for RCR vector replication exclusively in rapidly dividing prostate cancer cells.

III. KEY RESEARCH ACCOMPLISHMENTS

1. We have constructed replication-competent retrovirus (RCR) vectors containing suicide genes:
   - The smaller IRES-PNP gene cassette was successfully inserted just downstream of the viral *env* gene and its functionality confirmed by pro-drug activation assays.
   - Insertion of the larger HSV-tk gene was not pursued due to results from marker gene studies that indicate extreme instability of large insert sizes.

2. We have constructed RCR vectors targeted to prostate cancer cells by introduction of the probasin promoter into the retroviral LTR:
   - Probasin promoter elements were used to replace the endogenous viral promoter in the 3' LTR, using three different construct designs and two different versions of the probasin promoter.
   - Probasin-targeted RCR vectors containing the GFP marker gene as well as those containing the PNP suicide gene have been constructed.

3. We have established producer cell lines from TRAMP-C and LNCaP prostate cancer cells after initial RCR vector production by transient transfection of 293T cells.
   - The prostate cell-specificity of transcription driven by each probasin/LTR promoter construct was confirmed by luciferase assays in various prostatic and non-prostatic cell lines.
   - Androgen-inducible expression of the GFP marker gene from probasin/LTR-driven RCR proviruses in TRAMP-C and LNCaP producer cells was confirmed by FACS analysis.

4. We have tested the prostate cell specificity of replication and transgene expression by direct infection of probasin-targeted RCR vectors in prostatic and non-prostatic cell lines.
   - The prostate cell-specificity of replication driven by each probasin/LTR promoter construct was tested by FACS analysis in various prostatic and non-prostatic cell lines, and was confirmed to be highly prostate-specific and highly correlated with functional androgen receptor status.
   - The optimal probasin/LTR hybrid promoter design, showing the most robust replication kinetics in prostate cells upon androgen induction and no detectable background replication in non-prostatic cells, was determined to be the ACE-GFP-At design in which the synthetic ARR_2PB version of the probasin promoter is fused just upstream of the TATA box in the MLV LTR.
   - Prostate cell-specific killing by the PNP suicide gene expressed from the probasin/LTR-driven RCR vector ACE-PNP-At was confirmed by pro-drug activation assays.

5. We have confirmed prostate cancer cell-specific replication of probasin-targeted RCR vectors *in vivo* in tumor xenograft models.
   - Prostate cancer cell-specificity of the probasin-targeted RCR vector ACE-GFP-At was confirmed by *in vivo* fluorescence imaging, immunohistochemistry, and PCR analysis.

IV. REPORTABLE OUTCOMES

Peer-reviewed publications:

1. Logg, C. R., Logg, A. , Matusik, R., Bochner, B. H., Kasahara, N.
   Tissue-specific targeting of replication-competent retrovirus vectors by transcriptional regulation. *Journal of Virology*, 76: 12783-12791, 2002.

Abstracts and Presentations

1. Logg, A., Logg, C., Matusik, R. J., Bochner, B. H., Kasahara, N.
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2. Logg, C. R., Logg, A., Tai, C.-K., Park, J., Press, M. F., Bochner, B. H., Anderson, W. F., Cannon, P. M., Kasahara, N.
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   (Abstract selected for oral presentation at the 3rd Cold Spring Harbor Biotechnology Symposium on Vector Targeting Strategies for Therapeutic Gene Delivery, Cold Spring Harbor, New York, March 15-18, 2001.)

3. Logg, C. R., Logg, A., Matusik, R. J., Bochner, B. H., Kasahara, N.
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4. Logg, C. R., Logg, A., Tai, C. K., Wang, W., Anderson, W. F., Cannon, P. M., Bochner, B. H., Chen, T. Kasahara, N.
   Replication-competent retrovirus vectors targeted to cancer cells achieve highly efficient gene transfer to solid tumors *in vivo*.
   (Abstract selected for oral presentation at the 10th International Conference on Gene Therapy of Cancer, San Diego, California, December 13-15, 2001.)

5. Logg, C. R., Tai, C. K., Logg, A., Yoon, L. T. K., Lee, A. S., Anderson, W. F., Bochner, B. H., Cannon, P. M., Kasahara, N.
   Cell-specific transcriptional regulation and long-term genomic stability of replication-competent retrovirus vectors engineered for efficient transmission of exogenous genes.
   (Abstract selected for oral presentation at the 2002 Cold Spring Harbor Symposium on Retroviruses, Cold Spring Harbor, New York, May 22-24, 2002.)

6. Kasahara, N.
   Replicative retroviral vectors: *teaching an old virus some new tricks*.
   (Invited oral presentation at the Breakthrough Advances in Vector Technology session, 10th annual meeting of the European Society of Gene Therapy, Antibes Juan-les Pins, France, October 12-16, 2002.)

7. Logg, C. R., Logg, A., Matusik, R. J., Bochner, B. H., Kasahara, N.

   Transcriptional control of retrovirus vector replication to achieve highly efficient and tumor cell-specific gene transfer.

   (Abstract selected for oral presentation at the 4th Cold Spring Harbor Biotechnology Symposium on Vector Targeting Strategies for Therapeutic Gene Delivery, Cold Spring Harbor, New York, March 20-23, 2003.)

8. Kasahara, N.

   Targeting retroviruses: tumor-specific replication and gene transfer.

   (Invited oral presentation at the 4th Cold Spring Harbor Biotechnology Symposium on Vector Targeting Strategies for Therapeutic Gene Delivery, Cold Spring Harbor, New York, March 20-23, 2003.)

9. Kasahara, N.

   Replication-Competent Retrovirus Vectors for Suicide Gene Therapy of Cancer: *In Vivo* Therapeutic Efficacy, Tumor-Selectivity, and Safety.

   (Invited oral presentation at the 2nd International Meeting on Replicating Oncolytic Viruses for Cancer Therapy, Banff, Canada, March 26-30, 2003.)

10. Kasahara, N.

    Safety Considerations and Therapeutic Potential of Replication-Competent Retrovirus Vectors: Shifting the Risk vs. Benefit Paradigm.

    (Invited oral presentation at the Replicating Vectors Workshop, 6th Annual Meeting of the American Society of Gene Therapy, Washington D.C., June 4-8, 2003.)

Patents

Kasahara, N., Logg, C., and Anderson, W. F.

Gene Delivery System and Method of Use.

Provisional U.S. Patent Application No. 60/102,938
Conversion to full U.S. Patent Application No. 09/409,650

Cell lines, tissue or serum repositories developed

Murine TRAMP-C and human LNCaP prostate cancer-derived producer cells expressing probasin-targeted RCR vectors.

V. CONCLUSIONS

We have developed a stable, nondefective retroviral vector capable of high-level transduction both in culture and within solid tumors. The vector we developed consists of a wild type Moloney murine leukemia virus (MLV) with an internal ribosome entry site (IRES)-transgene cassette inserted between the 3' end of the *env* gene and the 3' long terminal repeat (LTR). We have now shown that incorporation of prostate-specific promoter elements into the viral LTR of this vector, in combination with its intrinsic inability to infect quiescent non-dividing cells, results in highly prostate cancer cell-specific replication in culture as well as in prostate xenograft tumors *in vivo*. Many of these results have recently been published (Logg et al, *J Virol* 2002; see Appendix).

There have been numerous published studies utilizing non-replicating retroviral vectors for *in vivo* gene delivery to tumors. In the large majority of these studies, the percentage of cells estimated to have been transduced was under 15%, despite the use of very high doses of vector or vector-packaging cells (Boviatsis *et al.*, 1994; Kruse *et al.*, 1997; Ram *et al.*, 1997; Short *et al.*, 1990; Smiley *et al.*., 1997). A recently reported clinical study of retroviral gene transfer into human brain tumors revealed that transduction of tumor cells occurred within only a few cell diameters of the tracts where vector-producing cells were injected (Rainov, 2000). In contrast, we estimate that the percentage of tumor cells transduced *in vivo* by our RCR vectors approached in some cases 100% (Logg *et al.*, 2001).

Since the general aim of cancer gene therapy is the destruction of malignant cells, the confinement of RCR vector spread to tumor cells would be of great utility. For this reason, we have taken steps toward engineering into the RCR vector the means to restrict vector replication to prostate cancer cells, by stringent transcriptional control using a highly prostate-specific promoter. Furthermore, by incorporation of a suicide gene as the therapeutic transgene to be delivered by these RCR vectors, infected tumor cells will be killed after administration of the pro-drug, thus also providing a mechanism to terminate any further virus spread. Through this project, we have achieved significant progress toward this goal, and we have developed the first reported examples of transcriptionally targeted replicating retrovirus vectors.

Through testing a series of different construct designs, we have optimized our probasin/LTR hybrid promoter, and we have now obtained an RCR vector that exhibits robust replication comparable to wild type virus in androgen receptor-positive prostate cancer cells, without detectable background expression in non-prostatic cells.

We have further been successful in incorporating the purine nucleoside phosphorylase (PNP) gene from E. coli as a suicide gene into our probasin-targeted RCR vectors, and we have confirmed highly prostate-specific cell killing from the probasin/LTR hybrid promoter after administration of its pro-drug MPDR.

We have further confirmed the specificity and efficiency of gene transfer by probasin-targeted RCR vector *in vivo* in animal models of prostate cancer. In future studies, we will apply the probasin-targeted PNP suicide gene RCR vector to these prostate cancer models in order determine its therapeutic effect *in vivo*.

The use of RCR vectors for cancer gene therapy will allow highly efficient gene delivery from a small initial inoculum due to the amplification inherent in the replicative process. The ability to specifically target RCR vectors to cancer cells, once achieved, will greatly enhance the efficacy and safety of this method. In particular, by achieving highly prostate-specific control, it may become possible to treat distant site metastases by systemic infusion of targeted RCR vectors, rather than by intratumoral injection. Interestingly, we now also have preliminary evidence that stable integration by MLV results in long-term persistence of viral infection, which follows cancer cells even as they metastasize to new sites, thus enabling multiple rounds of pro-drug administration to achieve further prolongation of therapeutic efficacy. Furthermore, RCR vectors thus confer permanent expression of viral proteins to the tumor cells with high efficiency; such stably and tumor-specifically expressed viral neoantigens can now be employed as a target for additional immunotherapeutic strategies. This makes the RCR vector system unique among the numerous oncolytic replicating virus systems now being tested as cancer therapeutic agents.

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V. APPENDICES

Please see attached publication.

(listed in Reportable Outcomes section above, and Final Report Bibliography section below).

VI. FINAL REPORT BIBLIOGRAPHY/PERSONNEL LIST (see also Reportable Outcomes section above)

Peer-reviewed publications:

1. Logg, C. R., Logg, A. , Matusik, R., Bochner, B. H., Kasahara, N.
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Tissue-Specific Transcriptional Targeting of a Replication-Competent Retroviral Vector

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The inability of replication-defective viral vectors to efficiently transduce tumor cells in vivo has prevented the successful application of such vectors in gene therapy of cancer. To address the need for more efficient gene delivery systems, we have developed replication-competent retroviral (RCR) vectors based on murine leukemia virus (MLV). We have previously shown that such vectors are capable of transducing solid tumors in vivo with very high efficiency. While the natural requirement of MLV infection for cell division imparts a certain degree of specificity for tumor cells, additional means for confining RCR vector replication to tumor cells are desirable. Here, we investigated the parameters critical for successful tissue-specific transcriptional control of RCR vector replication by replacing various lengths of the MLV enhancer/promoter with sequences derived either from the highly prostate-specific probasin (PB) promoter or from a more potent synthetic variant of the PB promoter. We assessed the transcriptional specificity of the resulting hybrid long terminal repeats (LTRs) and the cell type specificity and efficiency of replication of vectors containing these LTRs. Incorporation of PB promoter sequences effectively restricted transcription from the LTR to prostate-derived cells and imparted prostate-specific RCR vector replication but required the stronger synthetic promoter and retention of native MLV sequences in the vicinity of the TATA box for optimal replicative efficiency and specificity. Our results have thus identified promoter strength and positioning within the LTR as important determinants for achieving both high transduction efficiency and strict cell type specificity in transcriptionally targeted RCR vectors.

Defective vectors derived from oncoretroviruses possess a number of properties that have made them useful tools for therapeutic and experimental gene delivery, including low immunogenicity, integration into host cell DNA, a simple, well-characterized genome, and ease of production. It has become clear from a large number of studies, however, that defective retroviral vectors are generally incapable of efficient transduction when administered in vivo (14, 25, 33–35, 39, 40, 49). Although certain gene therapy applications, such as those in which transduction confers a selective advantage to target cells (3, 24) or those requiring only relatively low levels of expression of a secreted product (23), may not require high gene transfer efficiency, others, such as cytoablative strategies for genetic cancer therapy, generally require high transduction efficiency (45).

As a means for attaining more efficient gene delivery, we have constructed a series of replication-competent retrovirus (RCR) vectors derived from murine leukemia virus (MLV) (26). We demonstrated that an RCR vector containing an internal ribosome entry site (IRES)-green fluorescent protein (GFP) cassette between the *env* gene and the 3′ untranslated region can transduce solid tumors in vivo with a level of efficiency much greater than that possible using standard defective retrovirus vectors (27). However, while replication competence imparts greatly enhanced gene transfer power to retroviral vectors, it also represents a potential increase in the risk of oncogenesis, particularly under conditions of severe immunosuppression (6). Confining viral replication exclusively to targeted cell types would be one way of minimizing this risk.

An approach to the targeting of defective retroviral vectors that has been utilized previously is that of replacement of the promiscuous transcriptional control elements within the U3 region of the long terminal repeat (LTR) with promoter sequences that are active only in certain cell types (5, 8, 18, 30). This strategy has consistently allowed the generation of vectors with transgene expression limited in a cell-type-specific manner. Applied to an RCR vector, such a targeting strategy could be used to target both vector replication and transgene expression. Thus far, however, there have been no studies examining the parameters critical for achieving efficient and tissue-specific transcriptional control of retrovirus replication.

In this study, we have characterized a series of RCR vectors targeted to prostate cells by replacement of various segments of the U3 region with sequences from the probasin (PB) promoter or a recombinant variant of the PB promoter. Transcription of the PB gene is regulated by androgen and is highly specific to prostate epithelium in rodents (20). A 450-bp fragment of the proximal rat PB promoter has been used to generate mice whose transgene expression is limited exclusively to the prostate epithelium (12, 13). In vitro studies have also demonstrated that although there is no human homolog for this gene, the proximal PB promoter retains high levels of activity and prostate specificity in human cells (22, 36).

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ARR_2PB is a synthetic variant of the proximal PB promoter that possesses levels of strength significantly greater than and prostate specificity equal to the parental promoter (47, 50) and has been used previously to target transgene expression in adenovirus vectors (1, 48). Our results demonstrate that both replication and transgene expression of an RCR vector can be transcriptionally targeted to particular cell types with high specificity and that efficient replication requires optimization of promoter strength and the retention of specific sequences within the native MLV promoter.

MATERIALS AND METHODS

Cell lines and virus production. LNCaP (16), MDA PCa 2b (31), DU145 (42), and PC-3 (19) human prostate carcinoma cells, NMU rat mammary carcinoma cells (4), and HeLa human cervical carcinoma cells (38) were obtained from the American Type Culture Collection. PC-3$(AR)_2$ cells (15) were kindly provided by Theodore Brown (University of Toronto). LNCaP, PC-3, and PC-3$(AR)_2$ cells were cultivated in RPMI 1640 medium with 10% fetal bovine serum (FBS). MDA PCa 2b cells were grown in Ham's F12K medium with 20% FBS and supplements as described previously (31). HeLa and NMU cells were cultivated in minimum essential Eagle's medium with 10% FBS. 293T cells (7) were grown in Dulbecco's modified Eagle's medium with 10% FBS. For luciferase assays, all media were formulated using FBS treated with charcoal-dextran (HyClone) to remove steroids. Vector stocks were produced by transfection of 293T cells with vector plasmid by using Lipofectamine Plus (Invitrogen).

Construction of plasmids. All vector plasmids described in this study were derived from pACE-GFP-dm, which contains a full-length amphotropic RCR vector encoding GFP and the cytomegalovirus (CMV) promoter in place of the 5′ U3 region. To construct pACE-GFP-dm, the CMV promoter was amplified from pEGFP-N1 (Clontech) and introduced into pAZE-GFP (26) in place of the 5′ U3 region by using overlap extension PCR (17). The resulting plasmid, pACE-GFP, was then subjected to site-directed mutagenesis (QuikChange kit; Stratagene) to introduce a *Pme*I site into the 3′ U3 region and remove the *Sac*I site in the *pol* gene, creating pACE-GFP-dm. The *Pme*I site was created at position −92 (relative to the transcription start site) by two point mutations, and the *Sac*I site was removed by the introduction of a silent point mutation. These mutations had no discernible effect on in vitro replication of the vector (data not shown). The introduced *Pme*I site and the remaining *Sac*I site at −30 in the 3′ U3 region are both unique in pACE-GFP-dm.

To create plasmids pACE-GFP-Pr and pACE-GFP-Ar, we used overlap extension PCR to replace the 3′ U3 sequences in pACE-GFP-dm, from the unique *Nhe*I site to the 5′ border of the R region, with the proximal rat PB promoter or the synthetic PB promoter variant ARR_2PB, respectively. Plasmids pACE-GFP-Pt and pACE-GFP-At were constructed by first amplifying the proximal PB promoter and ARR_2PB (from their 5′ termini to the 5′ borders of their TATA boxes) with primers that introduce a 5′-terminal *Nhe*I site and a 3′-terminal *Sac*I site. These PCR products were then used to replace a large stretch of the 3′ U3 region of pACE-GFP-dm (i.e., from the *Nhe*I site to the *Sac*I site just upstream of the MLV TATA box). Plasmids pACE-GFP-Pc and pACE-GFP-Ac were constructed by replacement of the 3′ U3 region of pACE-GFP-dm from the *Nhe*I site to the *Pme*I site with sequences of the proximal PB promoter or ARR_2PB PCR amplified from their 5′ ends to the 5′ borders of their CAAT boxes at position −48.

Luciferase reporter plasmids were generated by using pGL2-Basic (Promega). The entire length of each hybrid LTR was amplified from the vector plasmids by using a 5′ primer containing an *Sma*I site and a 3′ primer containing an *Mlu*I site. These products were then introduced into pGL2-Basic at the *Sma*I and *Mlu*I sites upstream of the luciferase cDNA.

Luciferase assays. Cells at 70 to 80% confluence in 6-well plates were transfected with 3 μg of luciferase reporter plasmid containing the hybrid LTR by using Lipofectamine Plus. Parallel cultures were transfected with plasmids pGL2-Control (Promega), which contains the luciferase gene under the control of the simian virus 40 (SV40) promoter and enhancer, or pGL2-Basic, which contains the luciferase gene but no promoter. Each culture was cotransfected with 2 μg of the β-galactosidase expression plasmid pCH110 to control for transfection efficiency. For cultures subjected to androgen induction, 5-α-dihydrotestosterone (DHT) was added to the media to a concentration of 1 nM at 24 h posttransfection. At 48 h posttransfection, reporter lysis buffer (Promega) was used to prepare extracts from the cells. Luciferase and β-galactosidase activities were measured with the Luciferase assay system and the β-Galactosidase enzyme assay system (Promega), respectively.

Infections with replicating vectors. LNCaP, MDA PCa 2b, HeLa, or NMU cells at 20 to 30% confluence in 6-cm-diameter dishes were infected with equal amounts of stock vector, corresponding to a multiplicity of infection of 0.05, in the presence of 4 μg of Polybrene/ml. At 3, 8, and 21 days postinfection, the cells were analyzed for GFP expression by using flow cytometry on a FACScan II apparatus (Becton Dickinson). Serial infections with the ACE-GFP-At virus were carried out by passaging vector taken from LNCaP cultures at day 21 postinfection through fresh LNCaP cultures every week thereafter for 7 weeks, using 20-fold dilutions of supernatant for each infection cycle. Genomic DNA from each infection cycle was isolated by using the GenomicPrep kit (Amersham-Pharmacia).

Vector titrations. The stock vector titer was initially determined by RNA dot blot analysis as described previously (32). Blots were probed with a *gag-pol* fragment of MLV labeled with [^{32}P]dCTP by using the Prime-It II kit (Stratagene), and titers were estimated by comparison to virion RNA of an ACE-GFP stock of known biological titer. Biological titers of ARR_2PB-targeted vectors were determined by exposing 20 to 30% confluent cultures in the presence of 4 μg of Polybrene/ml to dilutions of the vector produced by LNCaP, MDA PCa 2b, HeLa, or NMU cultures infected 30 to 32 days previously. The following day, the medium on the cells was replaced with fresh medium containing 50 μM azidothymidine (Sigma) to block the secondary spread of the vector. On day 3, the cells were analyzed by using flow cytometry to determine the number of cells expressing GFP. The titer of the vector produced from each infected cell type was determined by using the same cell type.

Southern blot analysis. Genomic DNA was isolated from infected LNCaP cells by using the GenomicPrep kit (Amersham-Pharmacia Biotech). The DNA was digested with *Nhe*I and *Sph*I and then repurified by ethanol precipitation. A total of 10 μg of each DNA was fractionated on an agarose gel and blotted onto a Zeta-Probe nylon membrane (Bio-Rad) by using a Stratagene Posiblot manifold. A *pol-env* fragment of pACE-GFP was random prime labeled and hybridized to the membrane in Stratagene QuickHyb hybridization buffer. The hybridized blot was visualized using a Storm PhosphorImager (Molecular Dynamics).

PCR analysis and sequencing. The 5′ LTR of the ACE-GFP-At vector provirus was amplified by PCR by using an upstream primer that binds the 22 5′-terminal nucleotides of the U3 region, a downstream primer that binds just upstream of *gag*, and Pfu polymerase (Stratagene). Genomic DNA from infected LNCaP cells or a molecularly cloned copy of the 5′ region of ACE-GFP-At was used as the template. The amplified products were separated by gel electrophoresis, and the predominant species were extracted with a QIAquick gel extraction kit (Qiagen) and directly sequenced by using the downstream PCR primer.

RESULTS

Construction of hybrid PB-MLV LTRs. The proximal PB promoter contains elements that direct prostate-specific transcription, including a 148-bp sequence (from −244 to −96) called the androgen-responsive region (ARR; Fig. 1A). Within the ARR are two androgen receptor binding sites, which work cooperatively in the induction of promoter activity by androgens (21). A recently described synthetic variant of the proximal PB promoter, ARR_2PB, contains two copies of the ARR and exhibits more robust transcriptional activity than the wild-type (wt) promoter in vitro and in vivo (50). The PB promoter, ARR_2PB, and the MLV U3 region each contain CAAT and TATA boxes.

We constructed a series of six hybrid MLV LTRs incorporating sequences from the PB promoter. The hybrid LTRs were generated by replacement of the MLV U3 sequence (from the *Nhe*I site near the 5′ end to the CAAT box, TATA box, or transcription start site) with sequences from the wt PB promoter or ARR_2PB (Fig. 1B and C). Each of these LTRs was designed such that transcription would be initiated at the 5′ border of the R region, as occurs in wt MLV. LTRs Pr and Ar contain the wt PB promoter and ARR_2PB, respectively, with their transcription start sites fused precisely at the 5′

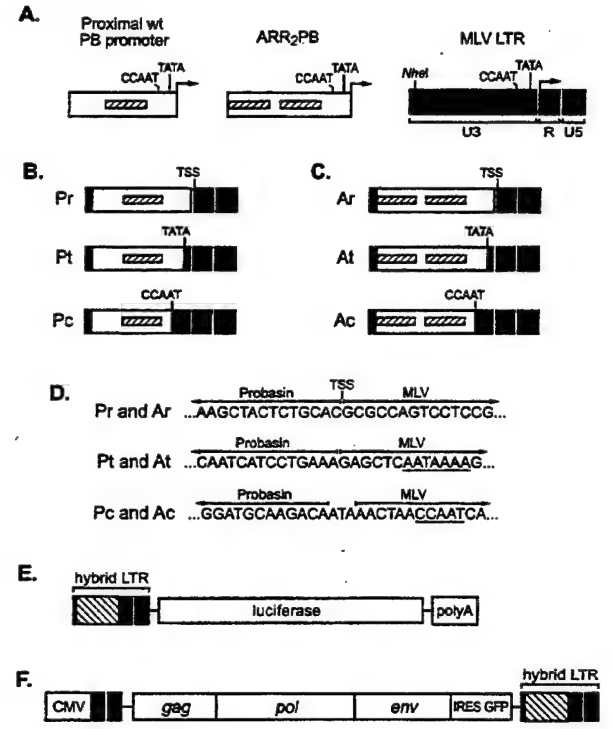

FIG. 1. Constructs used in this study. (A) Sequences used in generating hybrid LTRs. The proximal rat PB promoter (left) contains CAAT and TATA box homologies and an ARR (shown as a hatched box) important for androgen induction of transcription. ARR_2PB (center) is a synthetic variant of the PB promoter and contains two copies of the ARR. The MLV LTR (right) comprises the U3, R, and U5 regions. The transcriptional control sequences of MLV are located primarily in the U3 region, which also contains CAAT and TATA box sequences. (B) Hybrid LTRs containing the wt PB promoter. LTRs Pr, Pt, and Pc contain PB promoter sequences from position −383 to the transcription start site (TSS), TATA box, and CAAT box, respectively. (C) Hybrid LTRs containing ARR_2PB. LTRs Ar, At, and Ac contain ARR_2PB sequences from the 5′ end of the upstream ARR to the TSS, TATA box, and CAAT box, respectively. In each of the six hybrid LTRs, MLV U3 sequences from the *NheI* site to the TSS, TATA box, or CAAT box were replaced with the corresponding PB or ARR_2PB sequences. (D) Sequence details of the hybrid LTRs. Shown are the nucleotide sequences at the 3′ borders between the PB and MLV sequences. TATA and CAAT boxes are underlined. TSS, transcription start site. (E) Luciferase reporter constructs containing hybrid LTRs. (F) Structure of replication-competent MLV vectors containing hybrid LTRs. Each vector contains an IRES-GFP cassette positioned immediately downstream of the *env* gene and a 5′ LTR in which the U3 region was replaced by the CMV immediate-early promoter. The 3′ LTR is used to form the 5′ LTR during MLV replication. We therefore replaced the 3′ LTR of the RCR vector with the hybrid LTRs.

border of the MLV R region. LTRs Pt and At contain the wt PB promoter and ARR_2PB, respectively (from their 5′ ends to the 5′ ends of their TATA boxes), fused at the MLV TATA box. LTRs Pc and Ac contain the wt PB promoter and ARR_2PB, respectively (from their 5′ ends to the 5′ ends of their CAAT boxes), fused to the MLV CAAT box. A series of six reporter plasmids in which each of the hybrid LTRs controls expression of the luciferase cDNA were constructed (Fig. 1E).

Transcriptional activity of hybrid promoters in prostate and nonprostate cells. LNCaP and MDA PCa 2b prostate carcinoma, HeLa cervical carcinoma, and NMU mammary carcinoma cells were transiently transfected with the reporter plasmids containing the hybrid LTRs to assess transcriptional activity and cell type specificity. Transfections were carried out both in the presence and absence of androgen to evaluate the androgen inducibility of the hybrid LTRs. As a standard, a reporter plasmid containing the SV40 early promoter was used in parallel transfections.

In LNCaP cells, the three wt PB promoter-targeted LTRs and the SV40 promoter exhibited similar levels of strength in the absence of androgen (Fig. 2A). Upon androgen induction, activity levels of these hybrid LTRs increased three- to fourfold, while the SV40 promoter activity level remained unchanged. The ARR_2PB-targeted LTRs also exhibited, in the absence of androgen, a level of transcriptional strength similar to that of the SV40 promoter, although upon androgen induction, transcription from each of these LTRs increased roughly 1,000-fold. In MDA PCa 2b cells, all three wt PB promoter hybrid LTRs exhibited somewhat lower levels of strength than the SV40 promoter and were not induced by androgen (Fig. 2B). The LTRs containing ARR_2PB were significantly more potent in these cells than in those containing the wt PB promoter, driving luciferase expression to levels three- to fourfold higher than that by the SV40 promoter. Androgen also had no significant effect on transcription from the ARR_2PB-containing LTRs in MDA PCa 2b cells. The absence of induction in MDA PCa 2b cells was most likely due to the presence in these cells of a doubly mutated androgen receptor that exhibits greatly reduced affinity to DHT and increased affinity to other steroids compared to both the wt receptor and the singly mutated receptor present in LNCaP cells (51).

In the two nonprostate cell lines, the level of activity of each of the hybrid LTRs was much lower than that of the SV40 promoter, and androgen had no discernible effect on luciferase expression (Fig. 2C and D). Of the six hybrid LTRs, the two constructed using the CAAT box fusion design, Pc and Ac, exhibited the highest activity levels in HeLa and NMU cells. The higher nonspecific transcriptional activity level of these LTRs was most likely due to the retention of more of the MLV U3 region in these sequences than in the other LTRs. Surprisingly, the LTRs employing the TATA box fusion design consistently displayed the lowest activity levels in the nonprostate cell lines but levels of strength comparable to those of the other LTRs in the prostate lines. Use of the MLV promoter downstream from and including the TATA box in place of the corresponding region of the PB promoter therefore slightly improved, rather than impaired, prostate specificity. Taken together, these results show that, within the context of the hybrid LTRs, both the proximal PB promoter and ARR_2PB retain androgen inducibility and high specificity for cells derived from prostate epithelium.

Construction of replication-competent vectors containing hybrid LTRs. To assess the ability of the hybrid LTRs to support replication of a nondefective MLV and to impart prostate cell-specific replication, we replaced the 3′ LTR in an amphotropic, GFP-encoding RCR vector with each of these LTRs (Fig. 1F). The hybrid LTRs were introduced into the 3′ LTR position, as this copy is used as the template in the formation of both LTRs during reverse transcription. The vec-

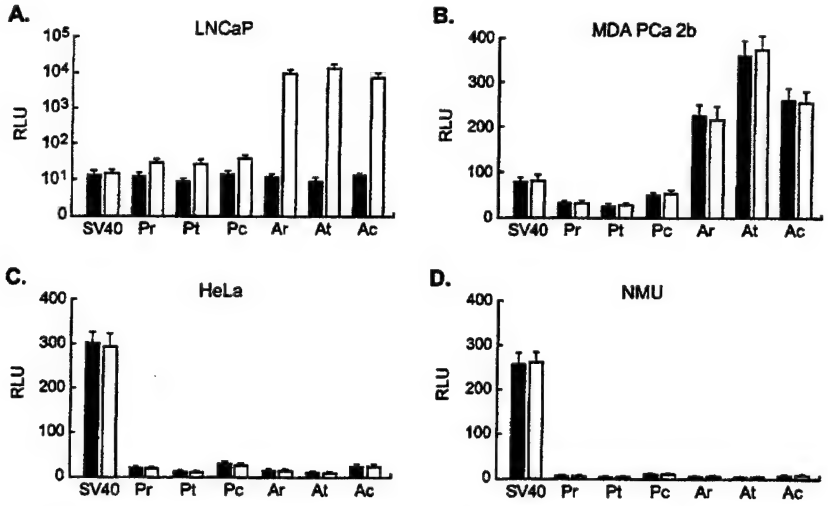

FIG. 2. Cell type specificity and androgen inducibility of transcription from hybrid LTRs containing wt PB promoter or ARR_2PB. Each of the hybrid LTRs was cloned upstream of the luciferase gene in plasmid pGL2 and transfected into two prostate (A and B) and two nonprostate (C and D) cell lines as indicated. Transcriptional activity was determined in the absence (black bars) and presence (open bars) of androgen. All cells were cultured in media containing serum that was charcoal stripped to remove endogenous steroids, and androgen induction was carried out by the addition of 1 nM DHT. Arbitrary relative light unit (RLU) levels were normalized for transfection efficiency, and the level determined for the promoterless control reporter plasmid in each cell line was assigned a value of 1. Results are the means obtained from at least three independent experiments. Error bars indicate standard deviations.

tor plasmid we used to construct the targeted vectors also contained the CMV promoter in place of the 5′ U3 region. In pilot studies, we found that when the 5′ LTR of the plasmid contained the wt U3, the 5′ LTR in the plasmid recombined during transfection with other plasmid molecules at the 3′ LTR to reconstitute an RCR vector with wt LTRs at both termini (data not shown). This resulted in contamination of the targeted vector preparation with an untargeted vector that exhibited a replicative advantage and eventually came to dominate the virus population. In contrast, no such revertants emerged in the vector-encoding plasmids after replacement of the U3 region of the 5′ LTR with the CMV promoter. Stocks of the targeted RCR vectors were generated by transient transfection of the vector plasmids, and their titers were initially determined by RNA dot blot analysis (data not shown).

Replication of targeted vectors in prostate and nonprostate cells. LNCaP, MDA PCa 2b, HeLa, and NMU cultures were inoculated with stock vector at a multiplicity of infection of 0.05, and vector replication was followed by flow cytometric analysis of the cultures at 3, 8, and 21 days postinfection. Although no exogenous androgen was added to these cultures, all media used during infections were made with nonstripped serum and thus contained endogenous androgen.

LNCaP cells infected with either ACE-GFP-Pt or ACE-GFP-Pc exhibited progressively increasing transduction levels over the 21-day period (Fig. 3A). The replication of these two vectors was significantly delayed relative to that of the untargeted parental vector ACE-GFP and spread to no more than half of the cells in each LNCaP culture after 21 days. In contrast, while the number of cells transduced by ACE-GFP-Pr was comparable to that by ACE-GFP-Pt and ACE-GFP-Pc by the 3-day time point, no spread was observed thereafter. ACE-GFP-Pt and ACE-GFP-Pc also replicated in MDA PCa 2b cells, although only very slowly, transducing approximately 10% of the cells in each culture after 21 days (Fig. 3B). ACE-GFP-Pr did not replicate in these cells beyond an initial low level of transduction. In HeLa and NMU cells, none of the three wt PB promoter-targeted vectors exhibited evidence of replication, while untargeted vector spread through both cell lines very efficiently. A small percentage (1.3 to 2.8%) of cells in HeLa and NMU cultures exposed to the wt PB promoter-targeted vectors expressed detectable levels of GFP at each time point, although these percentages were stable over several weeks and expression levels were extremely low (Fig. 3C and D and data not shown). As the vectors are targeted only at the transcriptional level, an initial low level of transduction by the vector stocks was not surprising. Of the HeLa and NMU cultures infected with the wt PB promoter-targeted vectors, those infected with ACE-GFP-Pc exhibited the highest percentage of cells expressing detectable levels of GFP, which is a finding consistent with the higher nonspecific transcriptional activity of the Pc LTR.

In LNCaP cells, both ACE-GFP-At and ACE-GFP-Ac replicated with kinetics markedly faster than the corresponding wt PB promoter-targeted vectors and only moderately slower than the untargeted vector (Fig. 4A), indicating that the greater transcriptional strength of ARR_2PB relative to that of the wt PB promoter enabled much more efficient vector propagation. Additionally, ACE-GFP-Ar, unlike ACE-GFP-Pr, was capable of replication in LNCaP cells, transducing on average approximately 20% of each infected culture after 21 days. Thus, the defect in replication of ACE-GFP-Pr was due at least in part to the insufficient strength of the wt PB promoter. MDA PCa 2b cells supported levels of replication of the three ARR_2PB-targeted vectors that were similar but slightly lower overall (Fig. 4B), with ACE-GFP-Ar again spreading much more slowly than the other two. The slower spread of the targeted vectors in MDA PCa 2b cells compared to LNCaP cells is

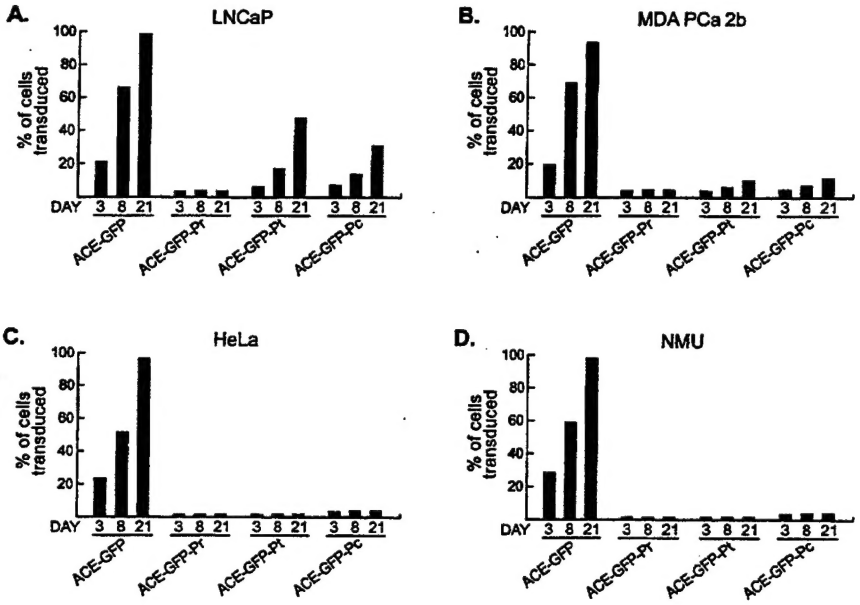

FIG. 3. Replication of wt PB promoter-targeted vectors in prostate and nonprostate cells. GFP expression in infected cells was assessed by flow cytometry on the indicated days postinfection. Results from infections of LNCaP (A), MDA PCa 2b (B), HeLa (C), and NMU (D) cells are shown. All cells were grown in nonstripped serum, and no exogenous androgen was added. Values are the means of three experiments.

consistent with the relative levels of transcriptional strength of the targeted LTRs in these cell lines. While the ARR_2PB hybrid LTRs mediated transcription levels roughly 10,000-fold higher than those of the promoterless control in LNCaP cells in the presence of androgen, transcription from these LTRs in MDA PCa 2b cells in the presence of androgen was by comparison 200- to 400-fold higher than that from the promoterless control (Fig. 2A and B). The disparity in the sequences of the androgen receptors in LNCaP and MDA PCa 2b cells may be responsible in part for these differences. Despite the greatly improved replication kinetics of the vectors containing ARR_2PB compared to that of the vectors containing the wt PB

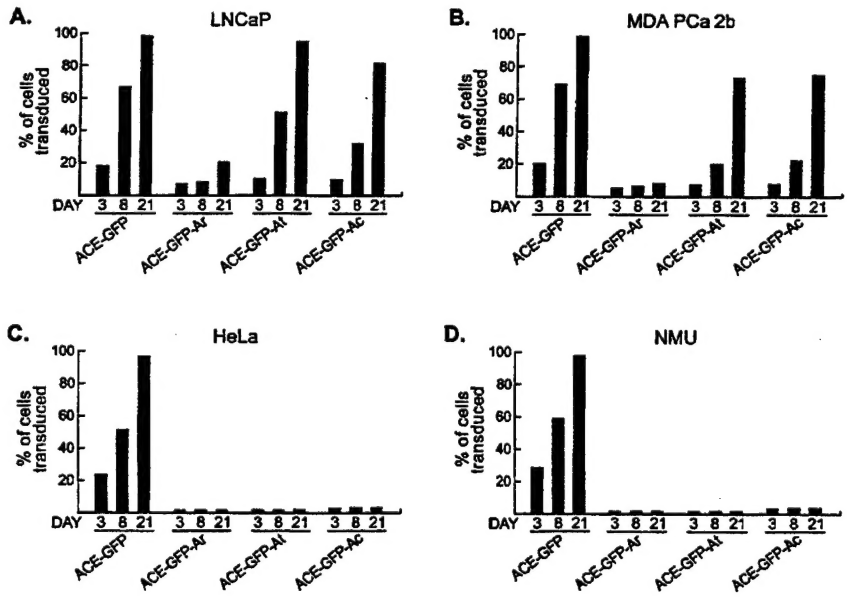

FIG. 4. Replication of ARR_2PB-targeted vectors in prostate and nonprostate cells. GFP expression in infected cells was assessed by flow cytometric analysis on the indicated days postinfection. Results from infections of LNCaP (A), MDA PCa 2b (B), HeLa (C), and NMU (D) cells are shown. All cells were grown in medium containing nonstripped serum, and no exogenous androgen was added. Values are the means of three experiments.

TABLE 1. Correlation of transcriptional activity of hybrid LTR At, replication of ACE-GFP-At, and presence of androgen receptor in human prostate carcinoma cell lines

Cell line	Androgen receptor expression	Efficient transactivation of hybrid LTR At[a]	Replication of ACE-GFP-At[b]
LNCaP	+	+	+
MDA PCa 2b	+	+	+
DU145	−	−	−
PC-3	−	−	−
PC-3(AR)$_2$	+	+	+

[a] Cell lines denoted as positive exhibited luciferase expression levels 200-fold or more higher than those of promoterless control plasmids; cell lines denoted as negative exhibited levels less than 10-fold higher than those of promoterless controls. Data used were taken from transfections in charcoal-stripped serum containing 1 nM DHT.

[b] Determined by flow cytometric analysis of infected cells over a 21-day period following exposure to stock vector.

promoter in prostate cells, GFP expression in infected HeLa and NMU cells remained restricted to an equally low, stable number of cells (Fig. 4C and D). Continued propagation of the HeLa and NMU cells infected with the ARR$_2$PB-targeted vectors for 6 weeks after inoculation did not result in an increase in the percentage of infected cells (data not shown).

Three additional prostate-derived cell lines were tested for their ability to support both transcription from the At LTR and replication of ACE-GFP-At. Two of these cell lines, DU145 and PC-3, do not express androgen receptor, while the third, PC-3(AR)$_2$, a PC-3 derivative, expresses androgen receptor from an episomal plasmid. Each of these cell lines was efficiently infected with untargeted vector (data not shown). However, neither DU145 nor PC-3 cells mediated either efficient transcription from the At LTR or replication of ACE-GFP-At (Table 1). In PC-3(AR)$_2$ cells, by contrast, transcription from the At LTR was highly efficient and ACE-GFP-At was capable of replication. Thus, we observed a strong correlation in prostate cell lines between efficiency of transcription from the At LTR, replication of ACE-GFP-At, and expression of androgen receptor, further corroborating the transcriptional specificity of this vector.

Determination of biological titers of targeted vectors. The titers of the three ARR$_2$PB-targeted vectors replicating in LNCaP, MDA PCa 2b, HeLa, and NMU cells were determined by using a flow cytometric assay. LNCaP cultures chronically infected by ACE-GFP, ACE-GFP-At, or ACE-GFP-Ac produced infectious vector at comparable levels of between 3×10^4 and 10×10^4 GFP-transducing units/ml (Table 2). In contrast, titers of ACE-GFP-Ar produced by the same cells were more than 10-fold lower than those of the other two targeted vectors, which is a finding consistent with the slower replication kinetics of this vector. ACE-GFP, ACE-GFP-At, and ACE-GFP-Ac each replicated on MDA PCa 2b cultures at titers roughly three- to sixfold lower than on LNCaP cells. In contrast, on the two nonprostate cell lines, the targeted and untargeted vectors were produced at very different levels. On NMU cells, average titers of ACE-GFP-At and ACE-GFP-Ac were 3 and 8%, respectively, of those of ACE-GFP. On HeLa cells, while the levels of ACE-GFP-At and ACE-GFP-Ac remained below the detection limit of the assay (30 GFP-transducing units/ml), ACE-GFP was produced at 1.8×10^3 GFP-transducing units/ml.

TABLE 2. Titers of ARR$_2$PB-targeted vectors

Vector	Titer (GFP-transducing units/ml) for cell type:			
	LNCaP	MDA PCa 2b	NMU	HeLa
ACE-GFP	9.9×10^4	2.9×10^4	1.0×10^4	1.8×10^3
ACE-GFP-Ar	3.0×10^3	ND[a]	ND	ND
ACE-GFP-At	4.8×10^4	7.6×10^3	300	<30
ACE-GFP-Ac	3.3×10^4	8.7×10^3	800	<30

[a] ND, not determined.

Structural analysis of proviral vector DNA. To confirm that the ARR$_2$PB vectors did not undergo genomic alterations during the infection of LNCaP cells, we analyzed integrated provirus from infected LNCaP cells by using Southern hybridization. For each of the vectors, only bands of the sizes expected for full-length proviruses were obtained (Fig. 5), demonstrating that the GFP transgene had been transmitted as part of the intact vectors. To assess the stability of the hybrid LTR sequences over multiple replication cycles, we subjected ACE-GFP-At to seven serial passages through LNCaP cultures and isolated genomic DNA from each passage. We utilized PCR to amplify the 5′ LTR from integrated vector provirus and sequenced the resulting products. The sequences of the PCR products from each of the seven vector passages were found to be identical to those obtained by PCR using an intact, molecularly cloned ACE-GFP-At provirus control (Fig. 6). The amplifications of both the control and experimental templates generated two products. One represented the full-length At LTR, and the other represented a variant form of the At LTR containing only one ARR. The presence of this variant in the control reaction product indicates that it was an artifact of the PCR. Thus, the integrity of the hybrid LTR of ACE-GFP-At appears to have been maintained through prolonged propagation of the vector. The retention of the prostate specificity of serially passaged ACE-GFP-At was further demonstrated by the absence of vector spread in NMU cells exposed to conditioned medium from each of the seven ACE-GFP-At-infected cultures (data not shown).

DISCUSSION

Virus vectors that replicate exclusively in targeted tissues would represent a valuable addition to the tools available for use in therapeutic and experimental gene transfer. In this study, we evaluated the possibility of targeting replication of a nondefective retrovirus vector to prostate carcinoma cells by replacement of transcriptional control sequences of the vector's native U3 region with sequences from the rat PB promoter. Our results demonstrate that this approach can be used to generate RCR vectors that replicate in a highly efficient and cell type-specific manner.

Much interest in replication-competent forms of a number of virus species for use in cancer therapy has arisen in recent years (37, 46). RCR vectors derived from oncoretroviruses such as MLV possess unique properties that may provide significant advantages for use in cancer gene therapy. First, most oncoretroviruses, including MLV, are not inherently cytopathic. Thus, the inclusion of a conditionally cytotoxic trans-

choroid plexus and was thus introduced into the LTR as a possible means of targeting viral replication to these cell types. The resulting virus, however, exhibited tissue tropism as broad as that of wt MLV. This was most likely due to the retention of a large stretch (−152 to −1) of transcriptionally active U3 sequence in the vector (11).

The results presented here help to define how a transcriptionally targeted MLV-based RCR vector can be optimally constructed. First, we have shown that the entire U3 region except for approximately 30 bp at each end can be replaced with sequences from a heterologous promoter without significantly reducing the vector's replicative ability. The sequence contained within this region therefore does not, at least in vitro, appear to play an important role in viral replication other than that of providing transcriptional regulation. Inclusion of any of part of this 380-bp region in a transcriptionally targeted vector appears to be unnecessary and would likely only serve to increase nonspecific transcription, as observed with the vectors and LTRs constructed using the CAAT box fusion design.

It is notable that replication of both of the vectors in which the targeting promoter was fused at the LTR's transcriptional start site was greatly impaired relative to that of the vectors having the CAAT or TATA box fusion designs. Given that the LTRs of all three designs exhibited very similar levels of transcriptional strength in prostate cells, these results imply that the 3′-terminal 30 bp of U3 are involved in some stage of viral replication other than transcription. In human immunodeficiency virus, the sequence of U3 downstream from the TATA box has been shown to play a role in 3′-end formation (10, 44). It has been proposed that the corresponding region of MLV might also play some role in 3′-end formation, through a hairpin loop formed in the viral RNA at the U3-R border (2). Our results provide evidence for some secondary, albeit unknown, function of this sequence.

Our results also demonstrate that a strong promoter is required to support efficient virus replication. Despite the fact that the Pt and Pc wt PB promoter-targeted LTRs possessed levels of transcriptional strength in LNCaP cells similar to that of the SV40 enhancer/promoter, the corresponding vectors only replicated with very slow kinetics. The use of the hybrid LTRs containing the much stronger ARR_2PB resulted in greatly improved replicative efficiency. This improvement in efficiency was not accompanied by an increase in replication in nonprostate cells.

The stability of the targeting sequences during vector replication would be of central importance in any consideration of the therapeutic use of a transcriptionally targeted RCR vector. Our results indicate that the sequence of an ARR_2PB-targeted LTR is retained over multiple serial vector passages through human prostate carcinoma cells. Additionally, the specificity of ARR_2PB-targeted vector for prostate cells was found to be unchanged after multiple passages, further confirming the stability of the hybrid LTR.

One potential use of a transcriptionally targeted RCR vector would be that of delivering a suicide transgene to tumor cells. Results published previously by Logg et al. (26, 27) demonstrate that RCR vectors containing insertions at the *env*-3′ untranslated region boundary can retain inserted transgenes over multiple cell-free passages and are stable enough to spread within and transmit inserted transgenes throughout solid tumors with very high efficiency. Delivery of a suicide transgene by an RCR vector into tumors should allow levels of transduction sufficient to achieve a potent antitumor effect following the administration of a prodrug. While the presence of a prodrug would result in the selection of variants of the vector from which the transgene has been deleted, if the prodrug is administered only after sufficient virus spread within the tumor has occurred, such selection pressure should not hinder the therapeutic effectiveness of this approach. Furthermore, the inclusion of a suicide transgene would provide a safety mechanism, as cells harboring the vector would be killed after exposure to the prodrug, thereby attenuating further vector spread. One motivation behind the present study was to examine whether vector replication could be restricted to particular target cell types so as to better confine the effects of a prodrug as well as the continued replication of potential transgene-deleted variants.

These studies indicate that replication of MLV can be directed with high specificity to particular cell types by replacement of the virus's nonspecific enhancer/promoter region with an exogenous promoter. An increasingly large number of cellular promoter sequences with various cell type specificities are becoming available, and many of these should be adaptable for use in generating RCR vectors capable of both highly efficient transduction and target-specific replication.

ACKNOWLEDGMENTS

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